Characterization of stromules visualized in transplastomic plants expressing green fluorescent protein (GFP)

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Introduction

The surface of plastids is bounded by an envelope consisting of two membranes. Most macromolecules, such as proteins and nucleic acids are not able to penetrate the inner membrane of the envelope. Thus, chloroplasts in general have been considered as discrete organelles. Green fluorescent protein (GFP) is a powerful tool to visualize the subcellular structures of organelles in living cells. Recently Köller et al. (1997) visualized the tubular projections emanating from chloroplasts in nuclear transgenic plants targeting GFP into chloroplasts. Sometimes tubules connect the chloroplasts with each other and GFP molecules are exchanged rapidly between the connected chloroplasts through the tubules. Stroma-containing protuberances were visualized by the electron microscopy in rice leaves preserved by high-pressure freezing (Bourett et al., 1999). Since the chloroplast tubules contain the extended stroma, they are named as 'stromules'. The stromules are rare in leaf mesophyll cells containing chloroplasts, but they are abundant in chlorophyll-free cells, such as root, petal and tissue culture, indicating that stromules are developmentally regulated.

Stromules may contribute to share molecules such as inorganic ions, metabolic intermediates, proteins and nucleic acids among plastids in the same cell. Genetic homogeneity of plastid genome may be mediated by stromules. Since stromules are sometimes associated with other cell organelles such as nuclei and mitochondria, they may function as a means of chemical communication between chloroplast and other cell compartments. However, the actual role of stromules in the functioning of chloroplasts and the molecular structure maintaining the stromules in shape remain unknown. We visualized chloroplast tubules in stable transplastomic plants expressing GFP via the plastid genome, providing a unique tool to study the exchange of protein molecules between chloroplasts and/or between chloroplasts and other organelles. Furthermore, we found that stromules could be induced by various external signals. Since stromules are rarely seen in leaf cells, it was difficult to investigate the function and morphology of this novel chloroplast structure. This finding enabled us to examine which cytoskeleton is involved in the maintenance of stromules in shape and their active movement.

Materials and Methods

Chloroplast transformation The *gfp* gene was modified by introducing mutations into a chromophore region (Phe^{64}Ser^{65} to Leu^{64}Thr^{65}). The tobacco *psbA* promoter core was cloned upstream of a *gfp* construct consisting of a SD signal; *gfp* and T*rps16* (Fig. 1).
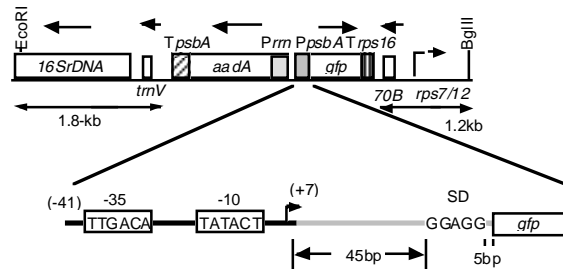

Figure 1 A gfp cassette driven by *psbA* promoter. P*psbA* indicates the position of the *psbA* promoter; *gfp* encodes the modified green fluorescent protein gene; P*rrn* indicates the position of the *16SrRNA* promoter; *aadA* encodes aminoglycocide 3"-adenylyl-transferase; T*psbA* referes to the e3'-UTR of the *psbA* gene. The *psbA* promoter core was connected with a gfp gene via a linker containing a synthesized SD signal.

The resulting *gfp* gene was cloned into a pKH5 plastid transformation vector designed following pRV112A (Zoubenko et al 1994). The pKH5 contains the *aadA* cassette (P*16SrDNA::aadA::*T*psbA*) excised from a plasmid pCT08 (Shikanai et al., 1998). Transformation of tobacco (*Nicotiana tabacum* cv. Xanthi) chloroplasts was carried out using a Biorad PDS1000He Biolistic gun at 1100 PSI following the method described by Shiina et al (2000). Transgenic shoots were selected on agar-solidified MS medium containing spectinomycin dihydrochloride (500 mg/L). Transgenic plants rooted on MS agar medium containing sucrose (3%) were used for observation with microscopes.

Confocal imaging Confocal imaging of cells was performed using a confocal laser-scanning microscope (μRadiance BioRad USA). The GFP was excited with the 488 nm of blue line and the chlorophyll was excited with the 568 nm line. These fluorescence images were collected in green or red channels, respectively. Serial optical sections were obtained at 0.5 μm intervals and processed using NIH image and PhotoShop (Adobe Systems Inc).

Protoplast fusion Mesophyll protoplasts were prepared by treatment with a solution containing 0.5 M mannitol, 2.5 mM MES (pH 5.7), 1 % Cellulase Onozuka and 0.5 % Macerozyme at 25 °C in the dark for 1 day. Wild-type protoplasts were mixed with protoplasts from the transplastomic plants and polyethylene glycol-induced fusion was carried out. The fused cells were incubated at 25 °C in the light.

Inhibitor treatment Cytochalasin D and colchicine were perchased from Sigma (USA) and dissolved in dimethylsulfoxide (DMSO) at 10 mM and 500mM, respectively. They were diluted with distilled water for use.

Results and Discussion

Chloroplasts are not connected with other cell organelles.

Tubular projections (stromules) emanating from the surface of chloroplasts have been visualized in nuclear transgenic tobacco, petunia and Arabidopsis cells targeting GFP into the stroma of chloroplasts (Koler and Hanson, 1997, Tirlapur et al., 1999, Arimura, et al., 2001). As shown in Fig. 2, chloroplasts are sometimes connected with each other through stromules. No chlorophyll autofluorescence was detected in the tubules, indicating that thylakoid membranes do not extend into them. The diameter of tubules was around 0.5 μm and their length was up to 10-20 μm. Stromules were often visible in bright-field images as well (panel B in Fig2).

It has been observed frequently that stromules are associated with other cell organelles, such as mitochondria or peroxisomes or nuclei. Close associations of stromules with other cell

organelles may facilitate the transport of molecules between chloroplasts and other cell organelles. Since we introduced a *gfp* gene into the chloroplast genome, it is possible to
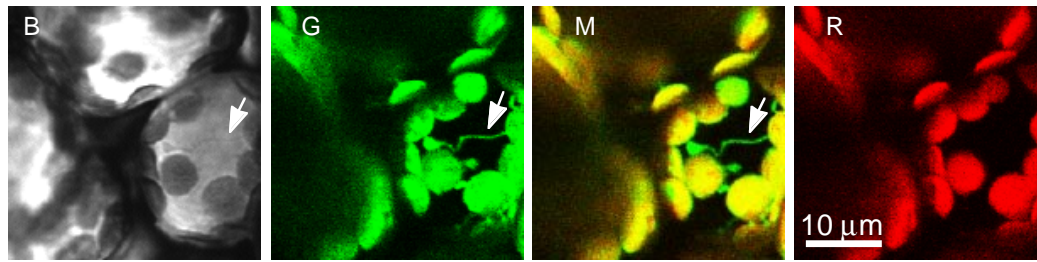

Figure 2 CLSM images of tobacco mesophyll cells expressing GFP in chloroplasts. Panel G shows GFP florescence (green channel), panel R red chlorophyll autofluorescence (red channel), panel M merged image of green and red channels, panel B bright-field image. Bar, 10μm. Arrows indicate a stromule.

examine readily whether chloroplast proteins can be transported to other organelles. In the transplastomic plants expressing GFP in chloroplasts, GFP fluorescence was localized in chloroplasts and stromules (Fig. 2). No GFP fluorescence was detectable in other cell organelles, such as mitochondria and nuclei. Over all, it was concluded that chloroplast is a discrete organelle and is not connected with other cellular compartments. However, these observations do not necessarily exclude the possibility that stromules are involved in the enhanced exchange of small molecules such as metabolic intermediates between chloroplasts and other cell compartments.

Are stromules involved in interchloroplasts transportation of macromolecules?

A rapid flow of GFP through the tubular connections between chloroplasts has been demonstrated by selective photobleaching of individual chloroplasts (Köhler et al 1997). It is expected that stromules are involved in the long distance transport of macromolecules. Transplastomic plants in which GFP was exclusively synthesized in chloroplasts enabled us to estimate the rate of GFP transfer among chloroplasts by fusioning protoplasts between the transplastomic and wild-type plants. Wild-type protoplasts were mixed with protoplasts from the transplastomic plants and polyethylene glycol-induced fusion was carried out. The fused cells were incubated at 25 °C in the light. At first, the two types of chloroplast with and without GFP fluorescence coexisted at the ratio of one to one in most of the fused cells. If tubular connections between chloroplasts were so frequent that all chloroplasts connected with each other via tubules, GFP fluorescence would distribute in all fused cell chloroplasts. Contrary to this, we found that the fraction of chloroplasts exhibiting GFP fluorescence in the fused cells did not increase significantly up to 3 days (Fig. 3). This strongly suggests that chloroplast connections are rare in most mesophyll protoplasts and exchange of endogenous proteins between chloroplasts does not occur frequently.
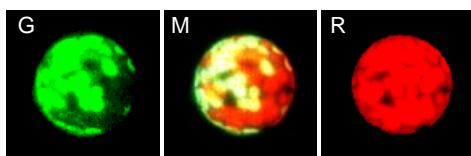

Figure 3 Fluorescent image of fused the protoplast with a conventional fluorescent microscope. Image G shows GFP fluorescence, image R chlorophyll fluorescence, image M merged image of green and red channels. Images were taken 2 days after the fusion of protoplasts.

Stromules were induced by various stresses.

Stromules were observed at a high frequency in tissues containing non-green plastids, such as root, petal and cultured cells. The shape of plastids in these tissues is irregular and they develop many long stromules (Köhler and Hanson, 2000). In contrast, stromules are rarely seen in leaf cells, such as mesophyll, trichome and guard cells. Thus, it is assumed that stromules are tissue-specific, and may be involved in the cell-specific functions. On the other hand, it has been pointed out that stromules were evident on most chloroplasts in some leaf cells, although only a few stromules were seen in the majority of leaf cells. Based on this observation, it was expected that stromules might be regulated by local environment as well. In order to identify the environmental signals regulating stromules, we treated leaf cells with various stresses. First, we examined the effects of drought stress on stromules in leaf cells. Small leaf segments were kept in the air for 10-15 min to apply water deficit stress and immediately dipped in water and observed by CLSM. As shown in Fig. 4, many long stromules were seen in leaf cells after drought stress. They did not disappear even if the tissue was kept in water for more
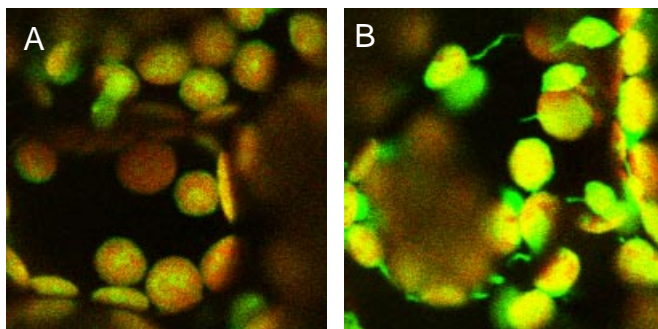

Figure 4 CLSM images of tobacco mesophyll cells before (A) and 60 min after drought stress. Merged images of the green and red images are shown. Image A shows projections of 10 optical sections taken at 0.25 μm intervals. Image B is a single optical section.

than 60 min, indicating the stromules were induced by drought signal and this signal was memorized for a long time. Furthermore, we found that stromules were also induced in leaf cells by osmotic or salt stresses. Immersion of leaf cell segments in a hypotonic solution resulted in plasmolysis in several minutes and accompanied by the induction of stromules (data not shown). Taken together, it is likely that membrane deformation or loss of turgor pressure may trigger the development of stromules on chloroplasts in leaf cells. Interestingly, stromules were also induced by mechanical stimulation (data not shown). The physiological role of the stress-induced stromules in the functioning of chloroplasts remains to be revealed. It should be noted that the induction of stromules by external signals in leaf cells would provide us an excellent tool to investigate the function and structure of stromules emanating from chloroplasts.

It has been suspected that high levels of GFP over expression in chloroplasts may result in abnormalities in chloroplast morphology and produce extended tubular structures artificially. However, stromules could be induced by various stresses, even if chloroplast GFP level was constant. Stromules were previously observed in non-transgenic plants by light microscopy. These facts suggest that stromules are not the artificial products of GFP over expression in chloroplasts.

Cytoskeletons are involved in the movement of stromules, but not the maintanance of the tubular structure.

Stromules in living cells moves dynamically. They continuously show rapid extension and retraction. It is expected that cytoskeletal network is required to maintain stromules in shape and support the stromule movement. Since chloroplasts are remnant of a free-living prokariote, cyanobacterium, it has been believed in general that chloroplasts lack the cytoskeleton, a complex network of actin, tubulin and intermediate filaments. Thus, it was expected that the morphology and movement of stromules depend on cytoskeletons in

cytoplasm. In order to characterize the cytosletal elements associated with stromules, we examined the effects of cytoskeleton inhibitors on the behaviour of stromules. As shown in Fig. 5, actively moving stromules were induced in leaf cells by drought stress. If this leaf segment was treated with 50 µM cytocharasin D, an inhibitor of the polymerization of actin, cytoplasmic streaming completely stoped within 5 min, indicating the destruction of microfilaments. Simultaneously, the movement of stromules stoped as well (Fig. 6). It is conceivable that stromule movement depends on actin microfilaments. However, the extended chloroplast tubules did not disappeared by cytochalacine D treatment. Stromules kept the same shape for a long period of several minutes, suggesting that the tubular structure is maintained by other cytoskeletal elements. Microtubules are unlikely to support the stromules, since colchicine scarcely affected both the movement and morphology of stromules (data not shown). Interestingly, tubular extensions were also observed in the isolated chloroplasts from leaf cells (Fig. 7). The stromules emanating from the isolated chloroplasts look like rigid extensions. Taken together, it is suggested that chloroplasts may contain their own inner rigid structure to keep stromules in shape.
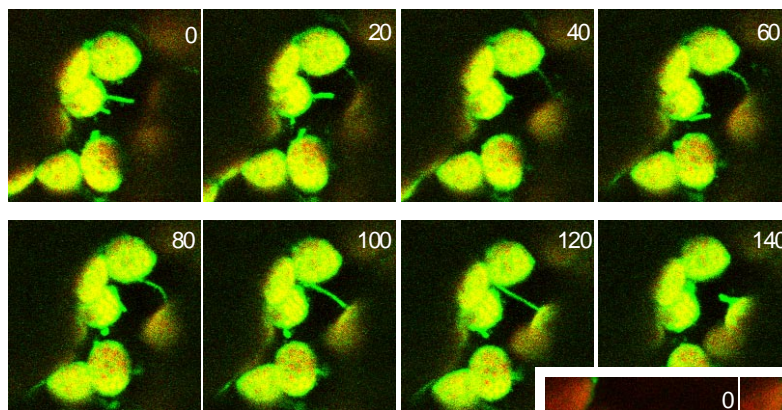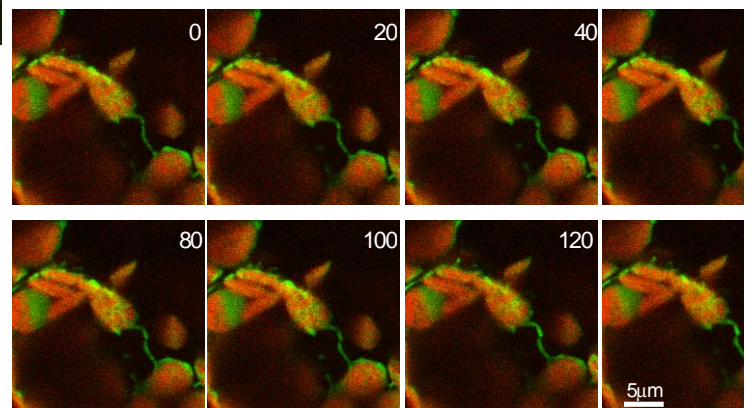

Figure 5 Stromules in a control mesophyll cell. Stromules were induced by drought stress. Images were taken at 20-s intervals and merged images of the green and red channels are shown. Numbers indicate the time in seconds.

Figure 6 Stromules in a mesophyll cell treated with cytocharasin D. Stromules were induced by drought stress. Leaf tissue was treated with 50µM cytochalasin D for 30 min. Images were taken at 20-s intervals and merged images of the green and red channels are shown. Numbers indicate the time in seconds.

Recently, it was demonstrated that chloroplast division protein, FtsZ forms a cytosleketon-like network in chloroplasts, when it was overexpressed (Kiessling et al., 2000). It is expected that FtsZ network is involved in the maintenance of plastid shape and flexibility. FtsZ-GFP fusion was also detected in thin stromules connecting
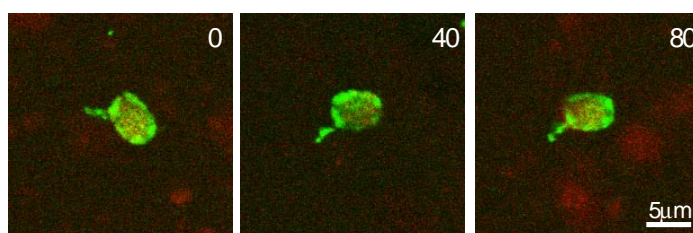

Figure 7 Stromule emanating from an isolated intact chloroplast. Chloroplasts were isolated from leaf mesophyll cells, which were treated with drought stress to induce stromules. Images were taken at 40-s intervals and merged images of the green and red channels are shown. Numbers indicate the time in seconds.

chloroplasts (Vitha et al., 2001). However, FtsZ could not be detected by immuno-fluorescence microscopy in the stromules. Further experimentation is required to elucidate the molecular structures supporting the extended stromules in shape.

Acknowledgements

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